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Shotgun Proteomic Analysis Unveils Survival and Detoxification Strategies by *Caulobacter crescentus* during Heavy Metal Exposure

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ABSTRACT. The ubiquitous bacterium *Caulobacter crescentus* holds promise to be used in bioremediation applications due to its ability to mineralize U(VI) under aerobic conditions. Here, cell free extracts of *C. crescentus* grown in the presence of uranyl nitrate [U(VI)], potassium chromate [Cr(VI)], or cadmium sulfate [Cd(II)] were used for label-free proteomic analysis. Proteins involved in two-component signaling and amino acid metabolism were up-regulated in response to all three metals, and proteins involved in aerobic oxidative phosphorylation and chemotaxis were down-regulated under these conditions. Clustering analysis of proteomic enrichment revealed that the three metals also induce distinct patterns of up- or down-regulated expression among different functional classes of proteins. Under U(VI) exposure, a phytase enzyme and an ABC transporter were up-regulated. Heat shock and outer membrane responses were found associated with Cr(VI), while efflux pumps and oxidative stress proteins were up-regulated with Cd(II). Experimental validations were performed on select proteins. We found that a putative phytase plays a role in U(VI) resistance and detoxification, and a Cd(II)-specific transporter confers Cd(II) resistance. Interestingly, analysis of promoter regions in genes associated with differentially expressed proteins suggests that U(VI) exposure affects cell cycle progression.

INTRODUCTION.

Anthropogenic activities have released significant amounts of heavy metals into the environment. According to the US Department of Energy (DOE), there are about 2 million acres in 30 states that are contaminated with uranium (U), and many DOE superfund sites are co-contaminated with other highly toxic heavy metals, such as chromium (Cr), lead (Pb), mercury (Hg), and cadmium (Cd).[1, 2] Extensive environmental pollution by heavy metals and radionuclides poses a great threat to ecosystems and human health.[3-5] The cleanup of heavy metal polluted sites is one of the most costly challenges faced by environmental scientists and governmental agencies.[2] The natural processes used by microbes to detoxify heavy metals in contaminated sites may provide cost-effective strategies for *in situ* remediation.[2, 6] Phylogenetically diverse bacteria have shown the ability to immobilize heavy metals with the potential to be used for bioremediation purposes.[7] A better understanding of the molecular mechanisms involved in heavy metal detoxification and immobilization in bacteria will improve the overall effectiveness of microbial bioremediation and help with the engineering of more effective microbes for *in situ* bioremediation.

Transcriptional and proteomic methods have been extensively used to examine microbes under heavy metal exposure and are powerful tools in identifying genome-level defense mechanisms by microbes. Studies have primarily focused on Cr(VI), Cd(II), and radionuclide stress by environmentally-relevant bacteria, including anaerobes, *e.g.* *Geobacter* spp.,[8, 9] facultative bacteria, *e.g.* *Shewanella* spp.,[10-13] *Acidiphilium* spp.,[14] and *Klebsiella* spp.,[15] and aerobes, *e.g.* *Pseudomonas* spp.[16-19] These studies revealed mechanisms of protection against oxidative stress, heat shock stress responses, and outer membrane defenses, including the induction of efflux pumps under various heavy metal exposures. In the case of Cr(VI) and U(VI), many genomic studies have examined anaerobic organisms that are able to detoxify these metals

by dissimilatory reduction.[20] However, few have examined aerobic organisms that lack the necessary reduction machinery, yet are still tolerant of the heavy metals.[20-23] These aerobic bacteria have significant potential to remediate contaminated sites at the aerobic zones and are the subject of this current study.

The ubiquitous, aerobic bacterium *Caulobacter crescentus* is able to survive under low-nutrient conditions[24] and has been shown to tolerate high concentrations of U(VI).[21] With its well-annotated genome and extensively developed genetic tools, *C. crescentus* is an excellent model system for studying heavy metal resistance.[21] In addition, *C. crescentus* serves as the model organism of cell cycle control in prokaryotes.[24] It exhibits asymmetric cell division in which two progeny with distinct morphological and regulatory features are produced: 1) a motile, chemotactic "swarmer" cell and 2) an immobile "stalked" cell, which is able to undergo subsequent cell divisions. Swarmer cells will differentiate into stalked cells in order to divide. The complex cell cycle of *C. crescentus* is mainly controlled by four master regulatory proteins, CtrA, DnaA, GcrA, and CcrM. These proteins undergo extensive post-translational phosphorylation and proteolytic processing in order to trigger various cell cycle progressions. The cellular concentrations of these regulators are carefully maintained over the course of the cell cycle during normal growth, and they are known to change drastically as a regulatory response to environmental stress and nutrient starvation.[25]

Here, we present proteomic profiling of *C. crescentus* CB15N under U(VI), Cr(VI), and Cd(II) exposure via a shot-gun proteomic approach. The goal of our study is to identify proteins differentially expressed under heavy metal stresses, and to compare the proteomic results with the already available whole genome transcriptional data.[21] To our knowledge, this is the first comprehensive proteomic description of *C. crescentus* subjected to heavy metal stresses. Comparisons between prior transcriptional data and proteomic data show many consistencies in

heavy metal stress response by *C. crescentus*. However, the proteomic results reveal several insights into cell cycle regulation under heavy metal stresses that can only be observed on the protein level, demonstrating the utility of combined whole-genome transcriptional and proteomic studies. Thus, our results provide further insight into the biochemical basis of heavy metal stress response by *C. crescentus* in the environment.

MATERIALS AND METHODS.

Bacterial strains, media, and materials. *Caulobacter crescentus* strain CB15N (ATCC 19089) was used in this study. All mutant strains of *C. crescentus* were prepared using standard double recombinant methods.[26] Cultures were maintained on rich medium PYE-agar (0.2% peptone, 0.1% yeast extract with 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, and 1.5% agar).[27] Liquid cultures were grown at 30 ^oC with shaking at 220 rpm in either 1) PYE; 2) M2G minimal medium containing 10 mM phosphate and 0.2% glucose as the sole carbon source;[27] or 3) modified M5G minimal medium at pH 7.0 containing 1 mM phytate as the sole phosphate source and 0.2% glucose as the sole carbon source.[28] All chemicals and enzymes were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Uranyl nitrate hexahydrate [$(UO_2)(NO_3)_2 \bullet 6H_2O$] was obtained from SPI Supplies (West Chester, PA). Heavy metal stock solutions at 100 mM were prepared in 0.1 N nitric acid for uranyl nitrate and Milli-Q water for potassium chromate (K_2CrO_4) and cadmium sulfate ($CdSO_4$). All metal stock solutions were sterilized by filtration through a 0.22 μm membrane prior to addition into growth media.

Growth conditions and heavy metal induction. A single colony of CB15N was used to inoculate 3 mL of M2G medium and the cells were grown overnight. Subsequently, 50 mL subcultures in fresh M2G medium inoculated with 1% (v/v) of the overnight culture were grown

for another ~24 h. The subcultures were diluted with fresh M2G medium to an optical density at 600 nm (OD_{600}) of 0.1 in a final volume of 500 mL. Cells were cultured until the OD_{600} reached 0.2 (early log phase), at which point uranyl nitrate (200 or 500 μM), potassium chromate (10 or 15 μM), or cadmium sulfate (7.5 μM) was added. Incubation was continued until the OD_{600} reached about 0.3 (mid-log phase) after about 3 h. The cells were then harvested by centrifugation at 20,000 x *g*, 4 ^{o}C for 10 min, washed twice with 1X PBS buffer, and the resulting cell pellets were saved at -80 ^{o}C until cell lysis and protein extraction.

Cell lysis and protein extraction. The protocol for cell lysis and protein extraction was adapted from previous work.[29, 30] In brief, frozen cell pellets were thawed on ice and re-suspended in PBS buffer. Cell suspensions were then normalized by OD_{600}, pelleted by centrifugation, re-suspended in 6 M guanidine hydrochloride with 10 mM DTT, and incubated at 37 ^{o}C overnight. After overnight incubation, the protein concentrations in the cell lysis solutions were assayed using the Bradford method and normalized. Aliquots were diluted by 6-fold in Tris buffer at pH 7.0, and then digested with sequence grade trypsin (Sigma-Aldrich) according to previously published procedures.[29, 30] The digested protein samples were centrifuged to remove the cellular debris and saved at -80 ^{o}C until LC-MS/MS analysis.

LC-MS/MS analysis. Proteomic runs were performed at the UC Davis Genome Center (Davis, CA). Digested peptides were analyzed by LC-MS/MS on a Thermo Scientific Q Exactive Orbitrap Mass spectrometer in conjunction with a Proxeon nanospray source and a Proxeon Easy-nLC II HPLC (Thermo Scientific, Waltham, MA). The digested peptides were desalted online using a 100 μm x 25 mm Magic C18 100 Å 5 μ reverse phase trap, and then separated using a 75 μm x 150 mm Magic C18 200 Å 3 μ reverse phase column (Bruker-Michrom,

Auburn, CA). Peptides were eluted using a 180 min acetonitrile gradient in 0.1% formic acid with a flow rate of 300 nL/min. An MS survey scan was obtained for the m/z range 300-1600 and MS/MS spectra were acquired using a top 15 method, where the top 15 ions in the MS spectra were subjected to High Energy Collisional Dissociation (HCD). An isolation mass window of 2.0 m/z was used for the precursor ion selection and a normalized collision energy of 27% was used for fragmentation. A 5 s duration was used for the dynamic exclusion.

Tandem mass spectra were extracted and charge state deconvoluted by Proteome Discoverer (Thermo Scientific). All MS/MS samples were analyzed using X! Tandem (GPM, thegpm.org; version TORNADO 2011.05.01.1). X! Tandem was set up to search the UniProt *Caulobacter crescentus* CB15N complete proteome sequence database (October 2012, 22650 entries), the cRAP database of common laboratory contaminants (www.thegpm.org/crap, 114 entries), and an equal number of reverse protein sequences assuming trypsin digestion, using a fragment ion mass tolerance of 20 ppm and a parent ion tolerance of 20 ppm. Iodoacetamide derivation of cysteine was specified as a fixed modification. Variable modifications included the deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, sulfonation of methionine, tryptophan oxidation to formylkynurenin, and acetylation of the N-terminus.

Protein identifications. Scaffold (version Scaffold_3.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. X! Tandem identifications required at least –Log(Expect Scores) of greater than 1.2 with a mass accuracy of 5 ppm. Protein identifications were accepted if they contained at least 2 identified peptides. Using the parameters above, the decoy false discovery rate (dFDR) was calculated to be 1.1% on the protein level and 0.0% on the spectrum level.[31] Proteins that contained similar

peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.[31]

Statistical analysis of relative protein abundance for heatmap illustrations. The raw spectral count data were first normalized by mean scaling such that each sample had the same total spectral counts. Secondly, we summarized protein counts for each condition by averaging the spectral counts of biological triplicates and calculating the Spearman correlation coefficients based on proteins that had a minimum of 10 spectra total across all conditions. As a result, 915 out of the 1279 proteins were included. Thirdly, unsupervised hierarchical clustering was applied to the Spearman correlation coefficients with Euclidean distance matrix and complete-linkage agglomeration. Heatmaps were generated using the heatmap.2 function in the R "gplots" package. Note, the data-processing procedure described above was only applied towards generating heatmap illustrations. In the differentially expressed protein analysis, the raw spectral count data were specifically modeled by Poisson regression (see below).

Poisson regression for testing differential expression. To compare spectral counts for different conditions, we modeled the data in a Poisson regression framework,[32] given the stochastic nature of spectral counts obtained by the mass spectrometer. We made pair-wise comparisons between each metal condition (test) and the control without metal. Using notation similar to Choi et al.,[32] we described the Poission regression model as follows. Consider p proteins and $2m$ replicates (*i.e.*, m replicates in each of the test and control conditions). For protein i, we assume the spectral counts of replicate j are observations from a Poisson distribution with expected count μ_{ij} for $j = 1,2,\ldots,2m$. Thus, the expected count matrix can be

expressed as:

(full model) $\log(\mu_{ij}) = \log(N_j) + \alpha_0 + \beta_{0i} + \beta_{1i} T_j$,

where N_j is the normalizing constant of replicate *j*, α_0 is the baseline abundance, and β_{0i} and β_{1i} are the protein-specific abundance and differential expression parameter for protein i, respectively. The treatment effect is defined by T_j where $T_j = 1$ if replicate j is the test condition and $T_j = 0$ if it is the control. For N_j, we use the total spectral counts across all identified proteins in replicate j to reflect the total abundance of all proteins in each MS/MS experiment.

The significance of the treatment effect β_{1i} was accessed via the likelihood ratio test,[32] which compares the log likelihood of the full model with that of the reduced null model (no treatment effect). The null model removes the treatment term resulting in:

(null model) $\log(\mu_{ij}) = \log(N_j) + \alpha_0 + \beta_{0i}$.

To address multiple hypothesis tests given thousands of proteins considered, we applied the false discovery rate (FDR) method[33, 34] to unadjusted p-values obtained by the likelihood ratio test for each protein. An estimated FDR of below 5% and at least a two-fold change in the estimated Poisson rates were required to declare a protein to be differentially expressed. Each protein was designated as +1 (up-regulated), -1 (down-regulated), or 0 (non-differentially expressed) (Table S1). Among the differentially expressed proteins, criteria were designated to reduce false positives. Proteins that were either poorly detected or were not detected in abundance in biological triplicates were labeled 0. Note that we developed in-house R scripts for the statistical data analyses described above; the R scripts are available upon request.

RESULTS AND DISCUSSION.

Concentration selection of metals and protein identification. In order to compare the proteomic data with previous transcriptional data, we chose metal concentrations that increased the doubling time by ~10% (Table S2), the same criteria used in the transcriptomic study.[21] Under these conditions, the cells are expected to experience metal stress, but their growth rate is not severely altered. Two concentrations were chosen for U and Cr in order to observe a dose-dependent effect. We note that the two U concentrations chosen were 200 and 500 μM, both of which increased doubling time by <3%. We did not choose higher U concentrations in order to avoid creating a phosphate-limited condition due to high amounts of uranium phosphate precipitation in the growth medium. All conditions were performed in biological triplicate to obtain statistically significant data.

In total, 1279 proteins were confidently identified by mass spectrometric measurements at the two unique peptide level across the 18 sample preparations (3 replicates x 6 conditions). The total numbers of proteins identified among triplicates and between different metal treatments were very similar, ranging from 947 to 982 (Table 1). As a first level of quality control for the shotgun proteomic analysis, the correlation between the expression profiles among replicate samples was assessed with respect to biological reproducibility. Total spectra counts for the identified proteins across the same treatment showed a Pearson correlation coefficient of > 0.9 for all data acquired, indicating good reproducibility among biological replicates. In addition, we observed that profiles for the two concentrations selected for U and Cr treatments clustered together (Figure S1), indicating that our approach was able to identify distinct proteomic features associated with each metal treatment.

Table 1 summarizes the number of differentially expressed proteins identified in each metal treatment. Greater than 100 proteins were differentially expressed under each condition,

including both up-regulated and down-regulated proteins. In general, Cr and Cd treatments identified similar numbers of differentially expressed proteins while U treatments identified fewer differentially expressed proteins compared to Cr and Cd. As we will discuss in subsequent sections, Cr and Cd may induce greater general and oxidative stress responses compared to U, resulting in greater numbers of differentially expressed proteins.

Comparison with transcriptome. We compared our proteomic results to the whole genome transcriptional profiling data in order to determine if translational and transcriptional profiles were consistent and to account for any differences. Protein levels can be regulated at different stages and the extent to which changes in mRNA levels are reflected in the level of proteins that they encode is an important consideration when evaluating the physiological relevance of stimulus-induced changes in transcript abundances. Since the down-regulated genes were only partially reported and had a >5 fold change cut off in the previous transcriptomic study, comparison was limited to the up-regulated gene products. Out of the total proteins up-regulated in the proteome under each U, Cr and Cd exposure, 25%, 18% and 32% were also found up-regulated in the transcriptome, respectively (Table 2). While there were similar numbers of genes/proteins up-regulated in the transcriptome and proteome under U stress, the numbers of up-regulated genes/proteins under Cr and Cd stress in the transcriptome were 2 to 3 fold higher than their respective proteome. In the case of Cr, the difference is likely caused by the higher metal concentration used in the transcriptomic study. Under all conditions, the lack of correspondence between transcript and protein levels could be due to the different timing of metal exposure. Considering that mRNA levels usually increase before an increase in protein levels, we collected cells after 3 h (about one doubling time) of metal exposure, as opposed to 30 min used during the transcriptomic study.[21] Post-transcriptional and post-translational

modifications as well as different rates of mRNA and protein degradation could also contribute to these discrepancies. In addition, the total numbers of proteins confidently identified in the proteome were only about a quarter of the genome, in contrast to the whole genome inquiry for transcriptomics, indicating an inherit limitation of the proteomic technique on the whole cell level. In later sections, we will compare transcriptomic and proteomic data sets for select proteins in greater detail.

Comparison between metal treatments. Quantitative analysis of the proteomic data revealed significant changes in protein expression caused by metal exposure. Out of the 1279 proteins identified, 325 proteins were up-regulated and 179 proteins were down-regulated significantly in at least one metal treatment. Among the 325 up-regulated proteins, 26, 55, and 35 proteins were uniquely up-regulated under U, Cr, and Cd stress, respectively, with 13 proteins (4%) shared among all three metals (Figure 1A). In contrast, there was more agreement among the different metals for the down-regulated proteins. Among the 179 down-regulated proteins, 36, 48, and 13 were uniquely down-regulated under U, Cr, and Cd conditions, respectively, with 40 common proteins (22%) shared among all three metals (Figure 1B). The high number of commonly down-regulated proteins reflects general strategies of cellular resource reduction/suppression induced by heavy metals.

The differentially expressed proteins for each metal treatment were clustered to reveal trends within the proteomic expression among the 5 metal treatments for each individual protein (Figure 1C). Consistent with clustering based on all proteins identified (Figure S1), clustering based on differentially expressed proteins revealed that samples from the two U treatments and the two Cr treatments were clustered together, respectively. The U treatments were most similar

to the control without metal treatment, probably due to fewer proteins differentially expressed under U.

Functional categorization of differentially expressed proteins. To gain insight into the biological context of relevant protein expression differences in response to metal stress conditions, we categorized all of the differentially expressed proteins, based on previously published COG categories for the *C. crescentus* CB15 proteome.[35] Our analysis revealed that differentially expressed proteins under a particular metal stress condition have a wide range of cellular functions (Figure S2). The majority of the differentially expressed proteins (28% for up-regulated proteins and 18% for down-regulated proteins) have no known function, highlighting that there are still many functionally unknown proteins that may play important roles in response to metal stress. The largest groups of annotated up-regulated proteins were involved in amino acid metabolism (10%), post-translational modification (9%), cell wall metabolism (9%) and inorganic ion transport and metabolism (7%). In contrast, the largest groups of down-regulated proteins were involved in energy production and conservation (14%), post-translational modification and protein turnover (10%), signal transduction mechanisms (7%), and inorganic ion transport and metabolism (6%).

Common proteins among metal treatments. The 13 commonly up-regulated proteins among all three metal treatments have a wide variety of predicted functions from cell wall and amino acid biosynthesis to signal transduction, metabolite transport, and energy production (Table 3). In particular, a two-component, signal-transduction response regulator, CC_1293 was found up-regulated 2 to 20 fold. The transcriptional targets of this particular response regulator have not yet been characterized. However given its up-regulation across all metal conditions tested, this

protein and its partner sensor histidine kinase CC_1294 may play an important a role in response to general heavy metal stress. Three proteins involved in amino acid metabolism were also found to be up-regulated under all conditions: an aminotransferase involved in aspartate metabolism (CC_1382), a D-cysteine desulfhydrase (CC_2032), and a peptidyl dipeptidase (CC_3702). The up-regulation of these proteins suggests that cells are degrading protein and metabolizing amino acids, possibly due to the presence of mis-folded proteins. Proteins involved in amino acid metabolism have also been shown to be up-regulated under Cr exposure in *Pseudomonas aeruginosa* and *Pseudomonas putida*[17, 19] as well as under Cd exposure in *Pseudomonas putida* and *Campylobacter jejuni.*[18, 36]

Proteins with the most significant down-regulation (FDR < 0.005 in almost all conditions) are shown in Table 3. Four proteins involved in aerobic oxidative phosphorylation (succinate dehydrogenase, cytochrome oxidases CydA and CoxB, and NADPH transhydrogenase PntB) were down-regulated, suggesting that the flux through the electron transport chain was significantly slowed down during all heavy metal stresses. In addition, *cydA* has been shown to have a cc_3 promoter motif, which is controlled by FixK, the global transcriptional regulator for respiration.[37] Thus, the down-regulation of CydA indicates reduced respiration under metal stresses in *C. crescentus*. The decreased flux through the electron transport chain may be a mechanism of reducing reactive oxygen species (ROS) generated during electron transport under oxidative stress conditions, similar to the response observed in *E. coli* under oxidative stress.[38, 39]

Chemotaxis protein McpJ was also down-regulated under all metal stresses. Various additional chemotaxis and cell motility proteins were found to be down-regulated under individual metal stresses. The transcriptional and/or translational down-regulation of chemotaxis and cell motility proteins was also found in *Shewanella oneidensis* MR-1 under Cr exposure[11, 13]

and in *Campylobacter jejuni* and *Pseudomonas putida* under Cd exposure,[18, 36] suggesting that the reduction of chemotaxis and cell motility is a common theme for bacterial heavy metal stress response. Furthermore, recent studies demonstrate that *Pseudomonas veronii* 2E, *Delftia acidovorans* AR and *Ralstonia taiwanensis* M2 species have reduced chemotaxis in fixed-bed reactors under various heavy metal stresses.[40] Heavy metals may interfere with chemoreceptors resulting in their down-regulation. Alternatively cells may sense a non-conducive environment for cell motility and thus reduce their chemotactic activities.

Proteins involved in protein translocation to the outer membrane (RsaD and SecD) as well as three transporters were found to be down-regulated under all stresses. These results indicate that cells may be experiencing membrane perturbation during heavy metal stress, giving rise to the re-organization of the cell wall. We also identified two down-regulated proteins (CC_1879 and CC_3636) that are involved in protein folding and are likely targeted to the periplasm based on SignalP 4.1 analysis,[41] suggesting a shift in the handling of protein conformation in the periplasm.

U specific proteins.

Protein up-regulated both translationally and transcriptionally. Proteomic data for up-regulated proteins under both U concentrations correlated very well with transcriptional microarray data (Table 4). Of the proteins commonly up-regulated under both concentrations of U, 40% (8 out of 21) were also found to be transcriptionally up-regulated. Notably, the two-component signal transduction systems (CC_1293/CC_1294 and CC_3303/CC_3304) were up-regulated as well as two transporters (CC_0224 and CC_2091) and a putative phytase (CC_1295). Precise deletion mutants of the individual two-component systems were previously prepared and showed no increased sensitivity toward U.[21] In order to test whether lack of U sensitivity in the single

mutants was due to complementation of the two systems, we prepared a double mutant in which both systems were deleted. The double mutant also did not exhibit increased sensitivity toward U during growth in PYE or M2G medium (data not shown), suggesting that the two systems are not redundant. We also prepared a precise deletion mutant of the ABC transporter CC_2091, which also failed to exhibit increased-sensitivity toward U (data not shown). The lack of a U sensitive phenotype in the tested mutants suggests that the respective genes do not play a direct role in U resistance under the conditions tested. Interestingly CC_2091 and CC_1304/CC_1305 contain cell cycle promoters motifs cc_1 and cc_2 respectively.[42] Expression of these proteins therefore may be related to altered cell cycle progression due to U stress rather than direct involvement in U detoxification. We will further discuss the effect of U stress on cell cycle progression in the following section.

Finally, the most highly up-regulated protein identified under U exposure was a putative phytase CC_1295. This protein was also found to be up-regulated under Cr exposure (Table S3). Phytases release inorganic phosphate from phytate, the main storage form of phosphate in plants, and provide a way for bacteria to obtain phosphate for metabolic functions.[43] Deletion of CC_1295 did not result in increased U sensitivity in PYE or M2G medium (data not shown). However, the mutant did exhibit increased sensitivity toward U in a modified M5G medium that includes 1 mM phytate as the sole phosphate source (Figure 2A). U(VI) is known to chemically precipitate with inorganic phosphate and bacteria are able to metabolize organic phosphates (*e.g.* glycerol-3-phosphate) in order to release inorganic phosphate, which precipitates with U as a detoxification mechanism.[22, 44, 45] Thus, the CC_1295 mutant, which is unable to obtain inorganic phosphate from phytate, may lack the ability to mineralize U through precipitation, resulting in its increased-sensitivity toward U.

U stress and cell cycle progression. Differentially expressed proteins under both U concentrations show that U exposure likely affects cell cycle progression. Most interestingly, we observed down-regulation of master cell cycle regulators DnaA and CtrA. DnaA activates replication initiation in *C. crescentus* and serves as a global transcription factor. In contrast, CtrA inhibits replication in swarmer cells, but also acts as a transcription factor for some 100 genes.[24] Consistent with the down-regulation of CtrA, we observed up-regulation of SigT, an ECF (extra-cytoplasmic function) sigma factor, known to regulate osmotic and oxidative stress responses.[46] SigT has been shown to contribute to the proteolysis of CtrA in carbon-starved swarmer cells.[25] The down-regulation of the constitutively-expressed response regulator CpdR, known to regulate the proteolysis of CtrA through its phosphorylation,[24] may be a result of the down-regulation of CtrA. We should note that depletion of DnaA and CtrA was not observed transcriptionally, most likely because both undergo post-translational proteolytic processing.[24, 25]

Down-regulation of both DnaA and CtrA has been observed in stationary phase and during nitrogen or carbon starvation by immunoprecipitation assays as well as proteomic analyses.[25, 47] Under carbon starvation, swarmer cells produced short, incipient stalks that did not fully develop. In stalked cells, cells remained in a pinched, pre-divisional state. Together, the results suggested that carbon starvation has the effect of blocking 1) full swarmer-to-stalked cell differentiation and 2) transition to S-phase and initiation of DNA replication, both of which occur when nutrients are sufficient. Cells were able to transition to an early-stalked state with the depletion of CtrA, but were not able to initiate replication due to the concomitant depletion of DnaA.

Here we hypothesize that U exposure promotes the formation of pre-stalked cells and affects DNA replication initiation, similar to that observed under carbon starvation. Firstly, U exposure showed the most significant down-regulation of cell motility and chemotaxis proteins,

including the flagellin protein FljM, compared to all other metals. Moreover, we observed dose-dependent depression with 3 more chemotaxis proteins down-regulated under 500 μM U compared to 200 μM U (Table S4). In addition, proteins involved in cell adhesion, including the pilus protein CpaD, were down-regulated under 500 μM U specifically. Chemotaxis and cell motility as well as pilus proteins are depleted during transition from swarmer to stalked cells.[48] The fact that these proteins are significantly down-regulated in a population predicts the prevalence of stalked or pre-stalked cells under U exposure. Additionally, the nucleoid-associated protein Smc, which normally aids in chromosome segregation among daughter cells,[49] was also up-regulated under U stress. The up-regulation of Smc may indicate growth arrest in the stalked-state, in which DNA is in a higher compaction state to inhibit replication and segregation.[25]

The striking similarities between carbon starvation and U stress suggest that *C. crescentus* may be experiencing nutrient-starvation- or stationary-phase- like conditions during U exposure. This response contrasts with responses to Cr and Cd (see below) where outer membrane and oxidative stress responses predominate, respectively. We did not, however, observe a significant change in growth rate with U under the experimental conditions tested, which may be due to insufficient changes in cell cycle proteins to cause a growth arrest. Experimental validations on the effect of cell cycle progression by U will be pursued in future studies.

Cr specific proteins. Commonly up-regulated proteins under both Cr concentrations reveal that heat shock/protein folding response and outer membrane response are the primary defenses against Cr in *C. crescentus* (Table 5). Heat shock response is primarily supported by the up-regulation of the RpoH sigma-32 factor involved in heat shock response as well as two small

heat shock proteins.[50] Additionally, three peptidyl-prolyl cis-trans isomerases were up-regulated, which are often involved in protein folding,[51] suggesting that these proteins are in higher demand to cope with unfolded or mis-folded proteins. Under 15 μM Cr, up-regulation of DnaJ, a major chaperone protein involved in heat shock response,[50, 52] was observed, indicating an increased heat shock response with increased Cr concentration (Table S5). Finally, a glutathione S-transferase (CC_2843) was up-regulated, which may be a response to oxidative stress or involved in disulfide bond formation during protein folding. The increase in abundance of heat shock related proteins in response to heavy metals has been reported previously.[10, 17-19, 36, 53] It is likely that ROS generated from Cr exposure results in the accumulation of mis-folded proteins, thus giving rise to heat shock response.[54]

Cr exposure resulted in the up-regulation of several TonB-dependent receptors involved in transport of various metabolites. Up-regulation of ABC transporters and Tol-like receptors was also observed as well a HlyD, type I secretion protein. Three out of the four TonB-dependent receptors identified contained a cc_13 promoter motif, suggesting that they are involved in a coordinated transcriptional response to Cr.[42] Interestingly, three TonB-dependent receptors with a cc_13 promoter motif were down-regulated under U and Cd conditions (Table S6), suggesting that Cr exposure has an outer membrane response distinct from U or Cd. Sulfate-specific transporters were not found to be differentially expressed, in contrast to what was reported for *Shewanella oneidensis* MR-1 under acute Cr stress.[13] Chromate is known to mimic sulfate and competitively bind to sulfate-binding proteins. The fact that we did not observe the up-regulation of sulfate-specific transporters suggests that the outer membrane proteins identified in this study likely are involved in a general outer membrane response by *C. crescentus*. Notably, however, sulfate-specific transporters were up-regulated primarily at >0.5 mM Cr exposure in *Shewanella oneidensis* MR-1; only general outer membrane transporters

were up-regulated at <0.5 mM.[13] Therefore, the concentration of Cr used in this study may not have been high enough to observe the sulfate-specific response. However, the large, general outer-membrane response under Cr exposure in *C. crescentus* suggests that Cr leads to a significant adaptation or reorganization of the cell membrane. Interestingly, the three peptidyl-prolyl cis-trans isomerases discussed above were found to contain signal peptide cleavage sites based on SignalP 4.1 analysis,[41] further suggesting periplasmic/membrane perturbation and reorganization. Cr stress has been linked with high amounts of lipid peroxidation supporting the presence of significant outer membrane perturbation.[54] Modifications of the cell wall are often associated with plant defense responses owing to its role as a physical barrier between the environment and the internal contents of the cell.[55]

We did not observe a large oxidative stress response in the presence of Cr in contrast to the transcriptional microarray data.[21] In particular, SodA, thioredoxins, glutathione-related proteins, and cytochrome c oxidases were not identified, which may be due to the lower detection limit of the shotgun proteomic approach. However, NepR, an anti-sigma factor for SigT,[56] the osmotic and oxidative stress regulator discussed earlier, was found to be down-regulated, suggesting some oxidative stress response may be turned on. The key factors in the discrepancy between the transcriptomic and proteomic data again are likely the different concentrations of Cr used in the studies as well the duration of metal exposure. Further, the oxidative stress response in *Shewanella oneidensis* MR-1 was found to predominate only at higher concentrations of Cr.[13]

Cd specific proteins. Proteomic data under Cd stress show detoxification mechanisms and oxidative stress response as the main modes of defense (Table 6). Detoxification mechanisms that were up-regulated under Cd stress included Ni/Co/Cd efflux pumps as well as defense

mechanisms toward arsenic and organic solvents, consistent with transcriptional data as well as other previous studies.[21, 57] Ni/Co/Cd specific efflux pump proteins NccB and NccC (CC_2721 and CC_2722) were up-regulated. To confirm the specificity of the Ncc efflux pump toward Cd, we prepared a precise deletion mutant of CC_2722. The CC_2722 mutant was found to be more sensitive to toxic levels of Cd, but not to U or Cr (Figure 2B), confirming that the Ncc system is a metal-specific efflux pump. Interestingly, Cd exposure also triggers the up-regulation of arsenate and organic solvent detoxification pathways. In the case of the arsenate resistance pathway, the transcription factor ArsR in *E. coli* (CC_1505 in *C. crescentus*) is known to closely resemble CadC in *Staphylococus aureus* and *Listeria monocytogenes* based on secondary structure predictions,[58] suggesting that the up-regulation of the arsenate pathway may be a result of non-specific binding of Cd to ArsR. It is unclear how Cd may trigger organic solvent detoxification pathways. However, organic solvent detoxification pathways are also up-regulated under 15 μM Cr (Table S5), suggesting that these pathways may be triggered as a general stress response, similar to previous observations in *Pseudomonas putida* during Cd exposure.[18]

C. crescentus exhibited the most apparent response to oxidative stress under Cd compared to the other heavy metals. First, several proteins involved in riboflavin biosynthesis (RibABDEH) were up-regulated as well as FolE (GTP cyclohydrolase I, responsible for the first committed step in tetrahydrofolate biosynthesis).[59] consistent with prior transcriptional data.[21] FolE has been shown to be one of the major targets of H_2O_2 damage in *E. coli*.[60] Therefore, the up-regulation of FolE is highly suggestive of oxidative stress. Up-regulation of riboflavin biosynthesis may be a result of damage to flavin-containing enzymes due to oxidative stress given the redox-active properties of flavins. Additionally, RibA (GTP cyclohydrolase II) has

been shown to be regulated by *soxRS* in *E. coli*.,[61] which is the major pathway for superoxide stress response.[62]

Second, Cd exposure revealed down-regulation of additional dehydrogenases in the electron transport chain for aerobic respiration (NuoF and SdhB), likely to further minimize ROS generated from electron transport proteins,[38, 39] and up-regulation of two oxidoreductases of unknown function (CC_2129 and CC_2269). The two up-regulated oxidoreductases may be a further result of maintaining redox balance in the cell, as oxidoreductases are known to react with a wide variety of redox active compounds.[63] Alternatively, their up-regulation may be a result of oxidative damage to their redox sensitive catalytic sites, which requires up-regulation of these proteins to compensate for the degraded activity.

Third, we observed an increase in Fe-S cluster biosynthesis, which is primarily supported by the up-regulation of NifU, a scaffold protein required for Fe-S cluster biosynthesis in nitrogenases.[64] The up-regulation of another HesB family protein, putatively involved in Fe-S cluster biosynthesis was also found. Interestingly, CysE, which catalyzes the rate-limiting step in cysteine biosynthesis, was up-regulated. Since the sulfurs in Fe-S clusters are well known to originate from cysteine, the up-regulation of cysteine biosynthesis may serve to support an increase in Fe-S cluster biosynthesis.[64, 65] Further, two TonB-dependent receptors were up-regulated, one of which is identified as a ferrichrome-iron receptor (CC_1778) and the other (CC_2660) a homolog to CirA receptors involved in Fe transport.[66] The increase in Fe transport proteins may also support increased Fe-S cluster biosynthesis. Interestingly, CC_1778 appears to overlap directly downstream of *sodA*, the superoxide dismutase that has been shown to be transcriptionally up-regulated under heavy metal stress.[21] The up-regulation of CC_1778 therefore may be a direct result of *sodA* up-regulation due to oxidative stress. The increased

biosynthesis of Fe-S clusters strongly suggests damage to Fe-S clusters in the cells, which further supports the presence of oxidative stress.

Finally, we found that five of the up-regulated proteins under Cd exposure, including FolE and RibD, contained an m_6 promoter motif that has been shown to be similar to the *rpoH* heat shock promoter in *C. crescentus*.[42] We also detected up-regulation of the unfoldase HslU, which is known to aid in protein turnover especially under heat shock stress.[67] It is possible that the heat shock response is triggered by the accumulation of proteins damaged by Cd induced oxidative stress.

Conclusions. The results of the present label-free shotgun proteomics study revealed a complex cellular response by *C. crescentus* to metal stress conditions and thus contribute substantially to our understanding of the still largely unknown mechanisms of heavy metal resistance and immobilization by aerobic bacteria. The results obtained indicate that different types of stress response can be elicited by different metal treatments at different concentrations. Proteomic changes in response to metal exposure revealed that cell survival and detoxification mechanisms resulted from enhancing a variety of cellular functions. Specifically, U response caused up-regulation of a putative phytase that enhances cell survival under U when phytate served as the sole phosphate source. Cd response caused up-regulation of a Cd-specific transporter that increases resistance to Cd. In contrast to U and Cd, Cr response appeared to be less specific, promoting alterations in outer membrane receptors and expression of heat shock-related proteins. Furthermore, the identification of cell cycle related promoter regions in multiple genes differentially expressed under U suggests that U stress response and cell cycle regulation are interconnected. The results of this study not only broaden our understanding of the fundamental aspects of metal stress response, but also provide insight into the roles of specific proteins in

metal detoxification. These insights might eventually pave the way to more effective bioremediation strategies for heavy metals under aerobic conditions.

ASSOCIATED CONTENT

Supporting Information. Table of change in doubling times under metal conditions. Tables of proteins differentially expressed specifically under U500 or Cr15. Tables of differentially expressed proteins shared among Cr and U, Cd and U, or Cd and Cr respectively. Excel file containing all identified and differentially expressed proteins. Cluster analysis based on Spearman rank correlation. COG distributions of differentially expressed proteins under metal conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS.

LC, liquid chromatography; MS, mass spectrometry; FDR, false discovery rate; U200, uranyl nitrate at 200 μM; U500, uranyl nitrate at 500 μM; Cr10, potassium chromate at 10 μM; Cr15, potassium chromate at 15 μM.

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TABLES AND FIGURES:

Table 1. Distribution of proteins identified in response to metal exposure. DE, differentially expressed; Up, up-regulated; Down, down-regulated; U200 and U500, uranyl nitrate at 200 μM and 500 μM respectively; Cr10 and Cr15, potassium chromate at 10 μM and 15 μM respectively; Cd, cadmium sulfate at 7.5 μM; control, no metal control.

	U200	U500	Cr10	Cr15	Cd	control
Total	947	964	970	971	969	982
DE	101	126	166	147	144	-
Up	27	48	65	78	66	-
Down	74	78	101	69	78	-

Table 2. Comparison of up-regulated proteins found in the current proteomic study with previous transcriptomic results.[21] The two numbers under U and Cr denote the number of proteins under each of the two concentrations tested.

	U	Cr	Cd
Proteome	27, 48	65, 78	66
Transcriptome	52	282	150
Common	12	14	21

Table 3. Proteins differentially expressed under all metal conditions. Relevant promoter motifs[42] are noted in parenthesis adjacent to the corresponding protein name. U200 and U500, uranyl nitrate at 200 μM and 500 μM respectively; Cr10 and Cr15, potassium chromate at 10 μM and 15 μM respectively; Cd, cadmium sulfate at 7.5 μM.

Genes	Log2 fold change					Annotation
	U200	U500	Cr10	Cr15	Cd	
Up-regulated						
Two-component signal transduction systems						
CC_1293	4.2	4.1	1.3[a]	1.6[a]	1.4[a]	DNA-binding response regulator
Amino acid transport/ metabolism						
CC_1382	>5	>5	>5	>5	>5	AatC, aminotransferase I, aspartate biosynthesis
CC_2032	1.9[a]	1.7[a]	1.9	1.6[a]	2.1	D-cysteine desulfurase
CC_3702	>5	>5	>5	>5	>5	Dcp, peptidyl-dipeptidase
Unknown						
CC_2247	>5	>5[a]	>5[a]	>5[a]	>5	Conserved hypothetical protein
Down-regulated						
Electron transport chain						
CC_0762 (cc_3)	< -5	< -5	< -5	< -5	< -5	CydA, cytochrome d ubiquinol oxidase subunit I
CC_3303	-3.9	< -5	< -5	< -5	< -5	PntB, NAD(P) transhydrogenase, beta subunit
CC_3407	< -5	-2.5	< -5	-1.7	< -5	CoxB, cytochrome c oxidase, subunit II
CC_3528	< -5	< -5	< -5	< -5	< -5	Succinate dehydrogenase, membrane anchor protein, putative
Cell motility/chemotaxis						
CC_3145 (cc_9)	< -5	< -5	< -5	< -5	-3.3	McpJ, methyl-accepting chemotaxis protein
Protein folding/ translocation						
CC_1879	-1.7	-1.2[a]	-2.1	-1.3	-2.5	DsbG-like, protein-disulfide isomerase
CC_1991	-4.2	-2.3	< -5	-1.2	-4.2	SecD, protein translocase subunit
CC_3636	< -5	< -5	< -5	< -5	< -5	Peptidyl-prolyl cis-trans isomerase, FKBP-type
S-layer biosynthesis						
CC_1008	< -5	< -5	< -5	-3.6	< -5	RsaD, RsaA secretion system, ATP-binding
Other transporters						
CC_1529[b]	< -5	< -5	< -5	< -5	< -5	Putative ABC transporter permease protein
CC_1520[b]	< -5	< -5	< -5	< -5	< -5	Putative ABC transporter permease protein
CC_2664 (cc_6)	-2.7	< -5	< -5	-2.1	< -5	TonB-dependent lipoprotein

[a] FDR > 0.005
[b] Transcriptionally down-regulated under all conditions

Table 4. Proteins differentially expressed under both 200 and 500 µM uranyl nitrate. Relevant promoter motifs[42] are noted in parenthesis adjacent to the corresponding protein name.

Genes	Log2 fold change		Transcriptional log2 fold change	Annotation
	U200	U500		
Up-regulated				
Two-component signal transduction systems				
CC_1293	4.2	4.1	3.3	DNA-binding response regulator
CC_1294	3.3	3.5	2.7	Sensor histidine kinase
CC_1304 (cc_2)	5.4	5.3	3.3	PhoP, DNA-binding response regulator
CC_1305 (cc_2)	3.7	4.1	3.0	PhoQ, sensor histidine kinase
Possible extracellular activities				
CC_1295	6.1	6.1	2.4	3-phytase/6-phytase
Outer membrane response				
CC_0224 (cc_1)	3.3	2.7	3.4	TonB-dependent receptor, CirA-like for Fe transport
CC_2091 (cc_1)	1.8	1.3[a]	2.1	ABC-type antimicrobial peptide transport system, ATPase component
Cell growth and division				
CC_0373	>5[a]	>5[a]	NU	Smc, chromosome partition protein
CC_3475	>5[a]	>5	NU	SigT, RNA polymerase ECF-type sigma factor
Unknown function				
CC_1245	2.3	2.2	NU	Hypothetical protein
CC_3303 (cc_2)	>5	>5	2.9	Conserved putative membrane protein, PepSY-like peptidase (adjacent to *ompR*)
Down-regulated				
Cell motility/chemotaxis				
CC_0343 (cc_10)	< -5[a]	< -5[a]	ND	McpP, methyl-accepting chemotaxis protein
CC_0438	-3.0	-3.6	ND	CheD, chemotaxis protein
CC_0792 (cc_12)	-3.1	< -5	ND	FljM, flagellin protein
Cell cycle regulation				
CC_0008	-2.0[a]	< -5	ND	DnaA, chromosomal replication initiator protein
CC_0744 (cc_12)	-2.9[a]	< -5	ND	CpdR, DNA-binding response regulator
CC_3035	-1.5[a]	-2.7	ND	CtrA, cell cycle transcriptional regulator

[a] FDR > 0.005
NU = not transcriptionally up-regulated based on Hu et al.[21]
ND = not transcriptionally down-regulated based on Hu et al.[21]

Table 5. Proteins differentially expressed under both 10 and 15 µM potassium chromate.

Relevant promoter motifs[42] are noted in parenthesis adjacent to the corresponding protein name.

Genes	Log2 fold change		Transcriptional log2 fold change	Annotation
	Cr10	Cr15		
Up-regulated				
Heat shock/protein folding				
CC_0979	>5	>5	NU	PpiA, peptidyl-prolyl cis-trans isomerase A
CC_1582	2.4	2.4	NU	Peptidyl-prolyl cis-trans isomerase, cyclophilin-like
CC_1688	1.6	1.7	NU	Peptidyl-prolyl cis-trans isomerase, parvulin-like
CC_2258	2.0	2.3	NU	Small heat shock protein, Hsp20 family
CC_2843	>5	>5	NU	Glutathione S-transferase
CC_3098 (cc_1, m_6)	2.6	3.0	NU	RNA polymerase σ-32 factor (heat shock response)
CC_3592 (m_6)	2.7	3.4	NU	Small heat shock protein, Hsp20 family
Outer membrane response				
CC_0028	1.3	1.3	NU	TonB-dependent receptor
CC_0139 (cc_13)	4.2	4.1	NU	FecA, TonB-dependent receptor
CC_0683	1.7	1.3[a]	NU	HlyD, Type I secretion adaptor protein
CC_1515	1.1	1.2	NU	Putative PstS; ABC-type phosphate transport system
CC_2194 (cc_13)	1.9	1.8	NU	TonB-dependent hemin receptor
CC_2928 (cc_13)	1.5	1.6	NU	TonB-dependent receptor
CC_3228	3.3	3.6	NU	YbgF, Tol system periplasmic component
CC_3435	1.7	1.8	NU	Carboxy-terminal processing protease
Amino acid/carbohydrate metabolism				
CC_1507	2.4	2.7	NU	Glucose/Sorbosone dehydrogenase
CC_3032	1.6[a]	1.7[a]	1.3	Aminoacyl-histidine dipeptidase
CC_3485	1.4	1.6	3.6	Aspartate-semialdehyde dehydrogenase 2
Unknown				
CC_0027	>5	>5	NU	2-oxoglutarate-Fe(II) oxygenase superfamily
CC_0658	>5[a]	>5	2.0	Conserved metal-dependent amidohydrolase
CC_1035 (cc_11)	3.1	3.2	1.1	Conserved hypothetical protein
CC_1056	>5	>5[a]	1.1	Conserved hypothetical cytosolic protein
CC_2119	>5	>5	2.1	Conserved putative exported protein
CC_2193 (cc_13)	5.6	5.8	NU	Putative membrane protein
CC_2840	1.5	1.1	NU	Conserved aminopeptidase
CC_3061	4.8	4.9	NU	Putative membrane protein
CC_3392 (m_6)	2.1	2.0	NU	YceI-family periplasmic protein (polyisoprenoid binding)
CC_3584	1.6	1.5	NU	Conserved peptidase, M16 family
Down-regulated				
Pilus assembly				
CC_2944	-1.6[a]	-1.8	ND	CpaD, pilus assembly protein
General stress response				
CC_3476 (cc_7)	< -5	< -5	ND	NepR, anti-sigma factor
Outer membrane response				
CC_3461	-1.2	-1.1	ND	TonB-dependent receptor, CirA-like for Fe transport
Peptidoglycan biosynthesis				
CC_1984	< -5	< -5	ND	BamD, outer membrane protein assembly factor
Amino acid/lipid metabolism				
CC_0311	< -5	-2.3[a]	ND	Predicted carboxypeptidase, amino acid transport
CC_1677	< -5	< -5	ND	AcpP, acyl carrier protein
CC_3364	< -5	< -5	ND	ThrB, homoserine kinase

[a] FDR > 0.005
NU = not transcriptionally up-regulated based on Hu et al.[21]
ND = not transcriptionally down-regulated based on Hu et al. [21]

Table 6. Proteins differentially expressed under 7.5 µM cadmium sulfate. Relevant promoter motifs[42] are noted in parenthesis adjacent to the corresponding protein name.

Genes	Translational log2 fold change	Transcriptional log2 fold change	Annotation
Up-regulated			
Detoxification mechanisms			
Cd-specific efflux pumps			
CC_2721	>5	4.4	NccC, nickel-cobalt-cadmium resistance protein
CC_2722	>5	5.2	NccB, nickel-cobalt-cadmium resistance protein
Arsenic resistance			
CC_1503	>5	2.3	ArsC, arsenate reductase
Organic solvent resistance			
CC_0478	2.7[a]	NU	Carboxymethylenebutenolidase, organic solvent degradation
CC_0490	1.5[a]	NU	Multicopper polyphenol oxidase (laccase, yfiH-like)
CC_3692	1.0	2.5	VacJ-like lipoprotein, Tig2 organic solvent resistance operon
CC_3694	3.5	2.3	Ttg2C, organic solvent resistance ABC transport system protein
CC_3758	2.2	NU	YsgA, carboxymethylenebutenolidase, organic solvent degradation
Protection against oxidative stress			
CC_0459 (m_6)	>5[a]	2.0	FolE, GTP cyclohydrolase 1
CC_0885 (m_6)	3.0	1.9	RibD, Pyrimidine reductase, riboflavin biosynthesis
CC_0886	3.7	2.1	RibE, Riboflavin synthase alpha chain, riboflavin biosynthesis
CC_0887	3.4	2.0	RibAB, GTP cyclohydrolase II, riboflavin biosynthesis
CC_0888	1.6	1.8	RibH, Riboflavin synthase beta chain, riboflavin biosynthesis
CC_1315	2.9	NU	Lactoylglutathione lyase
CC_3088	1.2[a]	NU	Glutathione S-transferase
CC_2129	1.3	2.2	NemA-like, NADH:flavin oxidoreductase
CC_2269	1.3[a]	NU	IorB, isoquinoline 1-oxidoreductase, beta subunit
Fe-S cluster assembly			
CC_0062	1.2	1.3	NifU, mitochondrial-type Fe-S cluster assembly protein
CC_2009	>5	1.1	HesB protein family, Fe-S cluster biosynthesis accessory protein
Heat shock/protein folding			
CC_0151 (cc_6, m_6)	>5	1.3	DegQ-like, trypsin-like serine protease
CC_3728	1.3	1.4	HslU, ATP-dependent protease, ATPase subunit (unfoldase)
Outer membrane response			
CC_1778	1.5	2.6	TonB-dependent ferrichrome-iron receptor (overlaps downstream of *sodA*)
CC_2660 (m_6)	>5	1.9	TonB-dependent receptor, CirA-like for Fe transport
Amino acid biosynthesis			
CC_1397	>5	NU	AroG, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, shikimate pathway for aromatic amino acids
CC_2138	1.6	NU	Methionine synthase I (cobalamin-dependent)
CC_2651	2.1	NU	CysE, serine acetyltransferase, cysteine biosynthesis
Cell wall/membrane biosynthesis			
CC_0301	>5	NU	LpxK, lipid A 4'-kinase, lipidA biosynthesis
CC_0351	>5	1.3	MipA-like, putative outer membrane protein, cell wall biosynthesis
CC_0414	>5	4.5	Fasciclin domain cell surface protein
CC_1759 (m_6)	1.2[a]	1.4	ABC transporter ATP-binding protein, lysophospholipase L1 biosynthesis
CC_2934	2.2[a]	NU	CMP-KDO synthetase, LPS biosynthesis
Others			
CC_0294	>5	NU	PhoB, phosphate regulon response regulator
CC_1889	>5	NU	Vitamin B12-dependent ribonucleotide reductase
CC_2029	1.1	NU	ThiC, phosphomethylpyrimidine synthase, thiamine biosynthesis

Continued on following page

Genes	Translational log2 fold change	Transcriptional log2 fold change	Annotation
Down-regulated			
Electron transport chain			
CC_1947	-2.2	ND	NuoF, NADH dehydrogenase I, F subunit
CC_3526	-1.2	ND	SdhB, succinate dehydrogenase, iron-sulfur protein
Outer membrane response			
CC_0362	-2.4	ND	PhnD, phosphonates ABC transporter, periplasmic
CC_1754	< -5	ND	TonB-dependent receptor
General biosynthesis			
Amino acid/ribosome			
CC_1360	< -5	ND	NusB, transcription termination factor, rRNA biosynthesis
CC_3737	< -5	ND	HisF, imidazoleglycerol-phosphate synthase protein (cyclase)
Purine			
CC_2497	-1.5	ND	PurQ, Phosphoribosylformylglycinamidine synthase I
Secondary metabolites			
CC_2961	< -5	ND	Limonene-1,2-epoxide hydrolase (terpene biosynthesis)

[a] FDR > 0.005
NU = not transcriptionally up-regulated based on Hu et al.[21]
ND = not transcriptionally down-regulated based on Hu et al. [21]

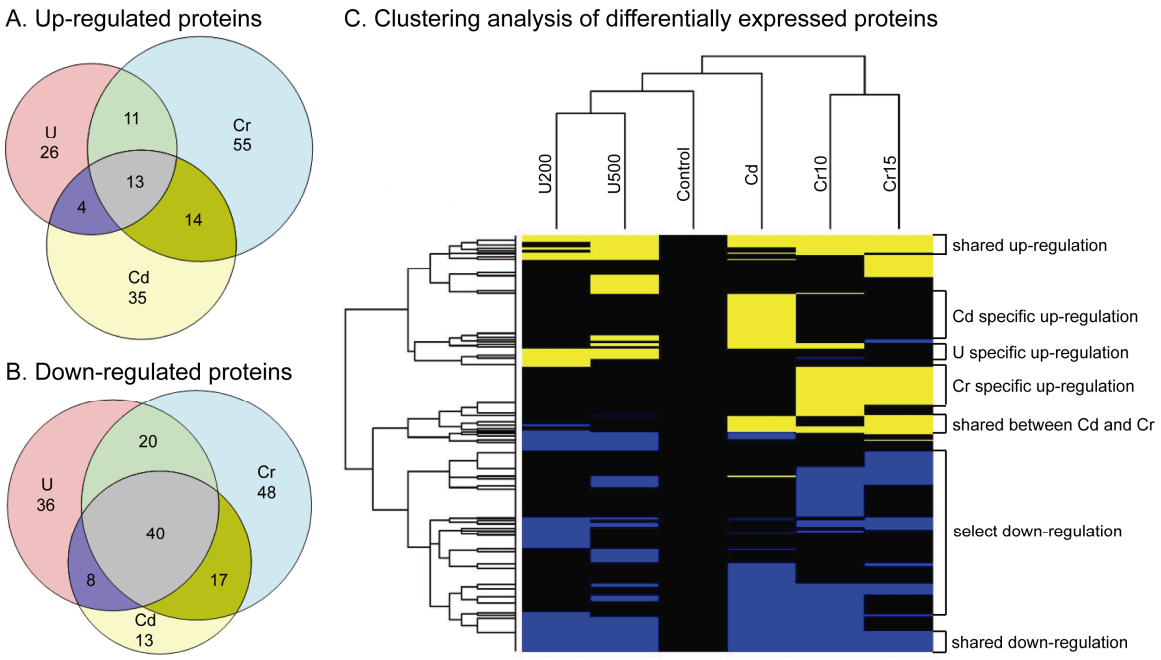

Figure 1. Distribution of differentially expressed proteins in response to metal exposure. A and B) Venn diagrams showing the number of up-regulated (A) and down-regulated (B) proteins in response to metal exposure. U, uranyl nitrate at 200 or 500 µM, Cr, potassium chromate at 10 or 15 µM, and Cd, cadmium sulfate at 7.5 µM. C) Heat-map representation of differentially expressed proteins (324 total) among the 5 metal treatments compared to the control with no metal. Metals are clustered from the left: uranyl nitrate at 200 µM (U200), uranyl nitrate at 500 µM (U500), no metal control (control), cadmium sulfate at 7.5 µM (Cd), potassium chromate at 10 µM (Cr10), and potassium chromate at 15 µM (Cr15). Proteins identified as up-regulated are shown in yellow, down-regulated in blue, and non-differentially expressed in black. Up-regulated proteins fall into 5 major clusters and down-regulated proteins fall into 2, identified to the right.

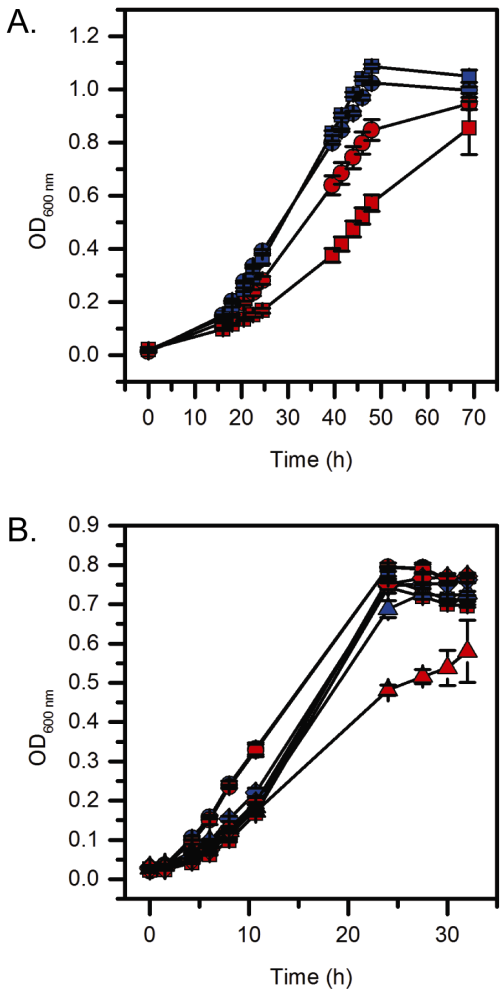

Figure 2. Growth of mutants in media supplemented with U, Cr, or Cd. A) Growth of wild type (blue) and CC_1295 phytase mutant (red) in modified M5G medium with 1 mM phytate supplemented with 100 µM uranyl nitrate (squares) or no metal (circles). Cells were pre-grown in PYE to saturation, washed once with M5G medium, and then inoculated into the indicated medium to an initial OD_{600} of 0.02. B) Growth of wild type (blue) and CC_2722 Cd transporter mutant (red) in PYE supplemented with 300 µM uranyl nitrate (squares), 15 µM potassium chromate (diamonds), 15 µM cadmium sulfate (triangles), or no metal (circles). Cells were pre-grown in PYE to saturation, and then directly inoculated into the indicated medium to an initial OD_{600} of 0.02. Error bars denote standard deviation from triplicate cultures.

GRAPHICAL ABSTRACT: FOR USE IN TOC ONLY